Changes of Lidocaine Concentration in the Jaw Bone and Oral Mucosal Tissue by the Addition of Adrenaline to Local Anesthetic

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局所麻酔薬へのアドレナリン添加による顎骨や粘膜の組織内リドカイン濃度の変化

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Abstract

Vaso-constrictive agents such as adrenaline are commonly added to local anesthetics for dental clinical use to enhance the effect. It is clear about the vaso-constrictive effect due to the addition of adrenaline to local anesthetic by measuring blood-flow volume or blood anesthetic concentration in oral mucosal tissue. However, there are no reports on the measurement of anesthetic concentration using samples directly extracted from the jaw bone and oral mucosal tissue. Consequently in this study, we clarify the effect of lidocaine concentration in the jaw bone and oral mucosal tissue by the addition of adrenaline to the local anesthetic lidocaine, through direct quantitative determination of lidocaine concentration in tissues.

Japanese white male rabbits (n=96) were used as test animals. After inducing general anesthesia with oxygen and sevoflurane, cannulation to the femoral artery was performed and arterial pressure was continuously recorded. Infiltration anesthesia was carried out using 0.5mL of 2% of lidocaine containing 1/80000 adrenaline in the upper jaw bone (A^+), and 0.5 mL of 2% of adrenaline additive-free lidocaine (A^-) under the periosteum. After a specified period of time (10, 20, 30, 40, 50 and 60 minutes), jaw bone, oral mucosa and blood were collected, and lidocaine concentration was directly measured by high-performance liquid chromatography (HPLC) method.

No significant differences in the change in blood pressure were observed either in A^+ or A^-. In both A^+ and A^-, the peak point of blood lidocaine concentration was observed 10 minutes after local anesthesia and decreased thereafter. At all time-points, concentration in A^+ was significantly lower than that in A^-. In the jaw bone and oral mucosal tissue, lidocaine concentration in A^+ was significantly higher than that in A^-, at all time-points. In both A^+ and A^-, no significant difference was confirmed between the jaw bone and oral mucosa.

By the addition of adrenaline to the local anesthetic, absorption of the local anesthetic into the blood was inhibited, maintaining a high concentration in the tissue. Adrenaline-induced vaso-constrictive effect was observed not only in the oral mucosa but also in the jaw bone.

和文抄録

歯科臨床で用いられている局所麻酔薬には，作用増強を目的としてアドレナリンなどの血管収縮薬が添加されていることが多い。その血管収縮効果は軟組織において，血流量の測定や，血漿中の局所麻酔薬濃度を測定することで明らかになっているが，実際の組織内の局所麻酔薬の濃度推移は明らかになっていない。そこで，本研究では局所麻酔薬リドカインへのアドレナリン添加が，顎骨や粘膜の組織内リドカイン濃度に与える影響を，直接組織内のリドカイン濃度を定量することで明らかにする。

実験動物として日本白色系雄性兎 (n=96) を用い，酸素，セボフルランで全身麻酔導入後，大腿動脈カニュレーションを行い，動脈圧を連続的に記録した。上顎骨に 8 万倍希釈アドレナリン添加 2%リドカイン（以下 A^+）と，アドレナリン無添加 2%リドカイン（以下 A^-）0.5mL を骨膜下に浸潤麻酔した。一定時間後 (10, 20, 30, 40, 50, 60 分後) に顎骨，粘膜および血液を採取し，リドカイン濃度を HPLC 法にて直接測定した。

局所麻酔時の血圧変動は A^+, A^- ともに有意差はなかった。血中リドカイン濃度は A^+, A^- ともに局所麻酔 10 分後に最高血中濃度となり，その後低下した。すべての時間で A^+は A^-より有意に低かった。顎骨，粘膜ともに，すべての時間で A^+のリドカイン濃度は A^-より有意に高かった。また，A^++，A^-ともに，顎骨と粘膜間では，組織リドカイン濃度に有意差を認めなかった。

局所麻酔薬にアドレナリンを添加することで，局所麻酔薬の血中への吸収は抑制され，高い組織内濃度が維持された。粘膜のみならず、顎骨内においても，アドレナリンによる血管収縮効果が確認された。

Introduction

Due to surgical stress in dentistry a stronger local anesthetic effect is needed not only for soft tissue, but also for hard tissue such as jaw bones. Currently, vaso-constrictive agents such as adrenaline are commonly added to local anesthetics for dental clinical use in order to inhibit bleeding from the surgical region through vaso-constriction and increase the effect of the local anesthetic by delaying the absorption of the local anesthetic and extending duration of effect[1-5]. It is clear about the vaso-constrictive effect due to the addition of adrenaline to local anesthetic. In most however, anesthetic concentration in tissue was indirectly considered by measuring blood-flow volume or blood anesthetic concentration[1, 6-11]. Regarding effect in bone, there are few reports about the vaso-constrictive effect due to the addition of adrenaline by such as the electrodialytical hydrogen clearance method and radioisotope measurement[12-14]. There are no reports on the measurement of lidocaine concentration using samples directly extracted from the jaw bone. Consequently in this study, we clarify the effect of lidocaine concentration in the jaw bone and oral mucosal tissue by the addition of adrenaline to the local anesthetic lidocaine, through direct quantitative determination of lidocaine concentration in tissues.

Materials and Methods

1. Animals

Ninety six Japanese white rabbits (body weight: 2.65 ± 0.3 kg, 16 weeks of age, male) (Nippon Bio-Supp. Center, Tokyo, Japan) were used. Animals were kept in an controlled animal room at 23°C and 60% humidity, and given free access to pellets (MF, Oriental Yeast, Tokyo, Japan) and drinking water (tap water) until the experiment day. This study was performed in accordance with the Animal Experiment Regulations of Ohu University (Permit No. 2013-49, 2014-29).

2. General anesthesia and experimental model

General anesthesia was induced by oxygen 5L/minute and 5% sevoflurane using anesthesia equipment for small animals (Soft Lander®, Shin-Ei Industries, Tokyo, Japan), and then a tracheotomy was performed, after which general anesthesia was maintained at oxygen 3L/minute and 3% sevoflurane. A cannula was inserted into the

femoral artery, and arterial pressure was continuously recorded throughout the experiment using a polygraph (Sanei Sokki, Tokyo, Japan) and a pressure transducer (Nihon Kohden, Tokyo, Japan) (Figure 1).

3. Infiltration anesthetic injection and excision of the tissue

Under general anesthesia, using quantitative syringe (Cartri-Ace®, Dentronics, Tokyo, Japan) with injection needle (27G, 0.40 x 19) (TERUMO NEEDLE®, TERUMO, Tokyo, Japan), 0.5 mL of 2% of lidocaine containing 1/80000 adrenaline (dental xylocaine cartridge® containing 1/80000 adrenaline, Dentsply Sankin, Tokyo, Japan) and 0.5 mL of 2% of adrenalin additive-free lidocaine (xylocaine injection polyamp 2%®, Astra Zeneca, Tokyo, Japan) was infused into the right maxillae, for 40 seconds, respectively. Injection site was the buccal side of the third molar on both sides (Figure 2). Subperiosteal infiltration anesthesia was performed by touching the needle tip to the jaw bone surface under the periosteum. The periosteum was dissected at specific time-points (10, 20, 30, 40, 50 and 60 minutes), and the injected maxillary and mucosa region (from the apical area of third molar to the infrazygomatic crest) were excised approximately 1g using rongeur forceps and stored at -80°C.

4. Measurement of the mean arterial pressure before and after local anesthesia injection

Even when under general anesthesia, pain stress changes arterial pressure[15], it can be reflected by polygraph. From the polygraphic arterial pressure data, 1/3 pulse pressure + diastolic arterial pressure was calculated as the mean arterial pressure (MAP), and changes in arterial pressure were assessed 10 and 20 seconds after infiltration anesthesia of 2% lidocaine with or without 1:80000 adrenaline.

5. Measurement of the blood lidocaine concentration

3mL of arterial blood was collected from femoral artery at specific time periods (10, 20, 30, 40, 50, and 60 minutes) after local anesthesia injection. The blood sample was centrifuged, and blood lidocaine concentration was measured by the enzyme multiplied immunoassay technique (EMIT) method[16-18].

6. Measurement of tissue lidocaine level

Bone and mucosa samples which were frozen were ground using a bone mill (TK-CM20S®, Tokken, Tokyo, Japan), suspended with 0.01 M boric acid at pH 9.18,

and homogenized for 2 minutes using a homogeniser (POLYTRON PT2100®, Kinematica, Switzerland). The supernatant (0.5 mL) was combined with 100 μL mexiletine (10 μg/mL) and then 5 mL of chloroform : methanol (8 : 2). After mixing, the solution was centrifuged at 3000 rpm (1000 G) for 10 minutes, and 3 mL of the organic layer was collected and dried under a reduced pressure at 40°C for 60 minutes using a rotary evaporator (EYELA®, Tokyo Rikakikai, Tokyo, Japan). The sample was then dissolved in 250 μL of the mobile phase (50 mM KH_2PO_4 : CH3CN = 4 : 1), stirred using a mixer, filtered, and applied to high-performance liquid chromatography (HPLC) (Jasco PU-2080 Plus®, JASCO, Tokyo, Japan) to measure the tissue lidocaine level, according to the method reported by Piwowarska et al[19]. Detailed HPLC conditions by Morota's report[20] is shown in Table 1. The typical chromatograms of lidocaine from rabbit bone and mucosa sample are shown in Figure 3. Tissue lidocaine data were converted to lidocaine level per g tissue.

7. Comparison statistics of data

The adrenalin addition group (A^+) and the adrenalin additive-free group (A^-) were compared in mean arterial pressure, blood lidocaine concentration and lidocaine concentration in tissue. Also the jaw bone and oral mucosa were compared in lidocaine concentration in tissue. In the statistical analysis, comparison within each group was performed by Friedman's test and comparison between groups by Mann-Whitney U-test. Statistical significance was set at $P < 0.05$.

Results

1. Variation in mean arterial pressure by local anesthetic injection

In A^+, mean arterial pressure of 78.0 ± 15.4 mmHg was obtained prior to local anesthetic injection. Obtained values were 71.3 ± 15.4 mmHg at 10 seconds after injection, and 81.1 ± 16.2 mmHg at 20 seconds. As a result, no significant difference was observed. In A^-, mean arterial pressure of 77.1 ± 15.0 mmHg was obtained prior to local anesthetic injection. Obtained values were 76.8 ± 14.7 mmHg at 10 seconds after injection, and 75.2 ± 15.3 mmHg at 20 seconds. As a result, no significant difference was observed (Figure 4).

2. Blood lidocaine concentration

In A^+, peak blood lidocaine concentration was 0.96 ± 0.17 μg/mL obtained 10 minutes after local anesthetic injection. Concentration decreased over time, and the obtained values at each time-point after injection were : 0.83 ± 0.14 μg/mL (20 minutes), 0.71 ± 0.13 μg/mL (30 minutes), 0.69 ± 0.13 μg/mL (40 minutes), 0.61 ± 0.12 μg/mL (50 minutes), 0.48 ± 0.13 μg/mL (60 minutes). In A^-, peak blood lidocaine concentration was 1.97 ± 0.40 μg/mL obtained 10 minutes after local anesthetic injection. Concentration decreased over time, and the obtained values at each time-point after injection were : 1.36 ± 0.16 μg/mL (20 minutes), 1.07 ± 0.20 μg/mL (30 minutes), 0.97 ± 0.15 μg/mL (40 minutes), 0.83 ± 0.17 μg/mL (50 minutes), 0.66 ± 0.16 μg/mL (60 minutes). At all time-points, blood lidocaine concentration in A^+ was significantly lower than that in A^-.

In A^+, the decrease of blood lidocaine concentration was gradual, while in A^-, a rapid decrease was observed after the 10-minute time point. The differences between the two groups were reduced gradually (Figure 5).

3. Lidocaine concentration in tissue

In A^+, the peak lidocaine concentration in jaw bone was 341.9 ± 154.5 μg/g obtained 10 minutes after local anesthetic injection. Concentration decreased over time, and the obtained values at each time-point after injection were : 277.6 ± 121.4 μg/g (20 minutes), 235.6 ± 97.9 μg/g (30 minutes), 163.8 ± 72.2 μg/g (40 minutes), 142.4 ± 9.8 μg/g (50 minutes), 71.5 ± 39.9 μg/g (60 minutes). In A^-, the peak lidocaine concentration in jaw bone was 114.5 ± 73.3 μg/g obtained 10 minutes after local anesthetic injection. Concentration decreased over time, and the obtained values at each time-point after injection were : 83.5 ± 59.6 μg/g (20 minutes), 51.3 ± 26.2 μg/g (30 minutes), 26.6 ± 19.3 μg/g (40 minutes), 22.6 ± 18.6 μg/g (50 minutes), 13.4 ± 14.6 μg/g (60 minutes). At all time-points, lidocaine concentration in jaw bone in A^+ was significantly higher than that in A^- (Figure 6).

In A^+, the peak lidocaine concentration in oral mucosa was 358.3 ± 111.5 μg/g obtained 10 minutes after local anesthetic injection. Concentration decreased over time, and the obtained values at each time-point after injection were : 303.5 ± 111.8 μg/g (20 minutes), 273.1 ± 124.8 μg/g (30 minutes), 194.1 ± 65.4 μg/g (40 minutes), 149.9 ±

28.1 μg/g (50 minutes), 118.3 ± 46.0 μg/g (60 minutes). In A^-, the peak lidocaine concentration in oral mucosa was 155.9 ± 128.0 μg/g obtained 10 minutes after local anesthetic injection. Concentration decreased over time, and the obtained values at each time-point after injection were : 81.0 ± 75.5 μg/g (20 minutes), 31.9 ± 19.3 μg/g (30 minutes), 15.0 ± 13.3 μg/g (40 minutes), 15.9 ± 18.3 μg/g (50 minutes), 4.6 ± 4.4 μg/g (60 minutes). At all time-points, lidocaine concentration in oral mucosa in A^+ was significantly higher than that in A^- (Figure 7).

In A^+, lidocaine concentration in jaw bone was lower than that in oral mucosa at all time-points, and no significant difference was observed between the concentration values of both groups (Figure 8). In A^-, however, lidocaine concentration in jaw bone was lower than that in oral mucosa only at the 10-minute time point. Values thereafter were reversed, and no significant difference was observed between the two concentration values of both groups (Figure 9).

Discussion

1. Variation in mean arterial pressure by local anesthetic injection

No significant difference in mean arterial pressure variation after local anesthetic injection was observed either in A^+ or A^-. Ichinohe et al.[21] reported that adrenaline increased cardiac output and decreased total peripheral vascular resistance, and that just one or two cartridges of 2% of lidocaine with 1/80000 adrenaline did not increase blood pressure in healthy adults. Troullos et al.[22] reported that the local anesthesia with 8 cartridges of adrenaline additive lidocaine in healthy adults increased heart rate and systolic blood pressure. Also Yamatsuta et al.[23] reported the influence of adrenaline on peripheral circulation in rabbits, and that 0.2 mL of 1/100000 to 1/500000 adrenaline had no influence on respiration or circulation. Morota et al.[20] reported that the strong pain or adrenaline overdose leads to the significant variation in blood pressure. In this study, 0.5 mL of 1/80000 adrenaline additive lidocaine was injected into rabbits, and no significant variation in mean arterial pressure was observed after injection. Therefore, it is estimated that the amount of the local anesthetics used in this study was appropriate, clinically. However, although not significant, a temporary decrease in mean arterial pressure was observed in A^+ after local anesthetic injection. As reported by Ichinohe et

al.[21], this is considered to occur due to β-adrenergic action, which causes a temporary decrease in peripheral vascular resistance along with mean arterial pressure, and α-adrenergic action, which is expressed in peripheral arteries immediately after the decrease and the increase in mean arterial pressure up to the value prior to injection.

2. Blood lidocaine concentration

At all time-points, blood lidocaine concentration in A^+ was significantly lower than that in A^-. Ito[10] reported that a significantly higher blood lidocaine concentration was observed in adrenaline additive-free lidocaine than in adrenaline additive lidocaine. Moreover, time to maximum concentration was also reported to be prolonged in adrenaline additive lidocaine, and the tendency towards absorption at almost the same speed was indicated in both groups after the 60-minute time-point. In this study, maximum concentration was observed at same time in both groups, while blood lidocaine concentration in A^+ was significantly lower than that in A^- at all time-points, and the significant difference was reduced after the 50-minute time-point. From the above results, inhibition of lidocaine migration from the tissue to the vessel due to the vaso-constrictive effect of adrenaline was demonstrated.

3. Lidocaine concentration in tissue

There are many reports on the vaso-constrictive effect due to the addition of adrenaline to local anesthetics. In most, however, concentration in tissue was indirectly considered by measuring blood-flow volume or blood lidocaine concentration[8, 10, 11], and there were no reports which directly measured local anesthetic concentration in tissue. Yasuda et al.[13] conducted quantitative analysis by autoradiography, using radioisotope ^{14}C- labeled lidocaine. According to the report, lidocaine rapidly disappeared from tissue when solely administered, while lidocaine concentration could be maintained in the injected areas by the addition of adrenaline. High concentration was also observed in adjacent tissue. However, depending on the mixture of radioisotopes, a different diffusion or disappearance rate from that of the original drug may occur. In this study, measurement of lidocaine concentration in tissue was possible by the HPLC method on lidocaine extracted from tissue. Vaso-constrictive effect by adrenaline could also be confirmed through measurement of the actual concentration in

tissue. Similar to the report by Yasuda et al.[13], significantly low lidocaine concentrations in both jaw bone and oral mucosal tissue were observed in A^- in this study as well. It is reported that this occurred due to the vasodilator effect of lidocaine[24], which is itself rapidly absorbed into blood vessels. This study provides evidence of such occurrence. It is also estimated that local blood-flow volume decreases and lidocaine absorption is inhibited due to the vaso-constrictive effects of adrenaline, and that this results in significantly high lidocaine concentration in tissue.

According to Ogawa et al.[25], in the thicker the cortical bone, the more time equired for local anesthetics to reach the bone and for lidocaine infiltration into the jaw bone. Local anesthetics injected under the periosteum infiltrates into jaw bone through the cortical bone until reaching the bone marrow, and is then absorbed into capillaries over time[26]. Since the insertion region in this study is under the periosteum and more lidocaine infiltrates into the mucosa than into bone through the cortical bone, a significantly low lidocaine concentration value in the jaw bone was estimated. However, although no significant difference was observed, there were still interesting results. In A^+, lidocaine concentration in the jaw bone was lower than that in the oral mucosa at all time-points. In A^-, however, lidocaine concentration in the jaw bone was lower than that in oral mucosa only at the 10-minute time point. Values reversed after that time point. It has been reported that a large amount of local anesthetics is rapidly absorbed into tissues with large blood-flow[1]. Therefore it is estimated, that the lidocaine concentration rapidly decreased in the mucosa where many blood vessels are located, and the concentration in the jaw bone decreased gradually. In other words, although the vaso-constrictive effect of adrenaline was observed in both the jaw bone and oral mucosa, it was indicated that the vaso-constrictive effect was slightly weaker in the jaw bone than in the mucosa since blood-flow is lower in the jaw bone than in the mucosa[27]. Future tasks include the further clarification of evidence of the vaso-constrictive effect of adrenaline occurring in jaw bone, by the measurement of adrenaline concentration in jaw bones, and the confirmation of actual histology.

Conclusion

By the addition of adrenaline to local anesthetic lidocaine, the effect on lidocaine

concentration in jaw bone and oral mucosal tissue was considered through direct quantitative determination of lidocaine concentration in tissue. As a result, high lidocaine concentration in tissue could be maintained by adding adrenaline to lidocaine. Adrenaline-induced vaso-constrictive effect was observed not only in the oral mucosa but also in the jaw bone. In oral surgery, this should be considered, along with duration of treatment and the surgical process.

Disclosure

The authors have declared no conflicts of interest.

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Figure legends

Table 1. Condition for HPLC analysis of lidocaine

Figure 1. Method of general anesthesia

General anesthesia was induced by oxygen 5L/minute and 5% sevoflurane, and then a tracheotomy was performed, after which general anesthesia was maintained at oxygen 3l/minute and 3% sevoflurane. A cannula was inserted into the femoral artery, and arterial pressure was continuously recorded throughout the experiment using a polygraph and a pressure transducer.

Figure 2. Location of infiltration anesthesia

0.5 mL of 2% of lidocaine containing 1/80000 adrenaline and 0.5 mL of 2% of adrenalin additive-free lidocaine was infused into the right maxillae, for 20 seconds, respectively. Injection site was the buccal side of the third molar on both sides (White arrow). Subperiosteal infiltration anesthesia was performed by touching the needle tip to the jaw bone surface under the periosteum.

Figure 3. One case of chromatogram in lidocaine concentration from oral mucosa in rabbit

Figure 4. Change of mean arterial pressure before and after infiltration anesthesia

No significant difference was observed.

Figure 5. Change of blood lidocaine concentration after infiltration anesthesia

At all time-points, blood lidocaine concentration in A^+ was significantly lower than that in A^-.

** $P < 0.01$ A^+ vs A^-

* $P < 0.05$ A^+ vs A^-

Figure 6. Change of lidocaine concentration in jaw bone after infiltration anesthesia

At all time-points, lidocaine concentration in jaw bone in A^+ was significantly higher than that in A^-.

** $P < 0.01$ A^+ vs A^-

* $P < 0.05$ A^+ vs A^-

Figure 7. Change of lidocaine concentration in oral mucosa after infiltration anesthesia

At all time-points, lidocaine concentration in oral mucosa in A^+ was significantly higher than that in .

** $P < 0.01$ A^+ vs A^-

* $P < 0.05$ A^+ vs A^-

Figure 8. Change of lidocaine concentration in jaw bone and oral mucosa in A^+ after infiltration anesthesia

Lidocaine concentration in jaw bone was lower than that in oral mucosa at all time-points, and no significant difference was observed between the concentration values of both groups.

Figure 9. Change of lidocaine concentration in jaw bone and oral mucosa in A^- after infiltration anesthesia

Lidocaine concentration in jaw bone was lower than that in oral mucosa only at the 10-minute time point. Values thereafter were reversed, and no significant difference was observed between the two concentration values of both groups.

Table 1. Condition for HPLC analysis of lidocaine

Pump	Jasco PU-2080 Plus
Detector	Jasco UV-2075 Plus
Sensitivity	0.001 AUFS
Column	TOSOH TSK-GEL ODS-100V 15 cm×4.6 mm
Column oven	Sugai V-630
Column temperature	40℃
Mobile phase	50 mM KH_2PO_4:CH_3CN=4:1
Flow rate	1.0 mL/min
Wave length	205 nm
Degasser	AZZOTA AG-12

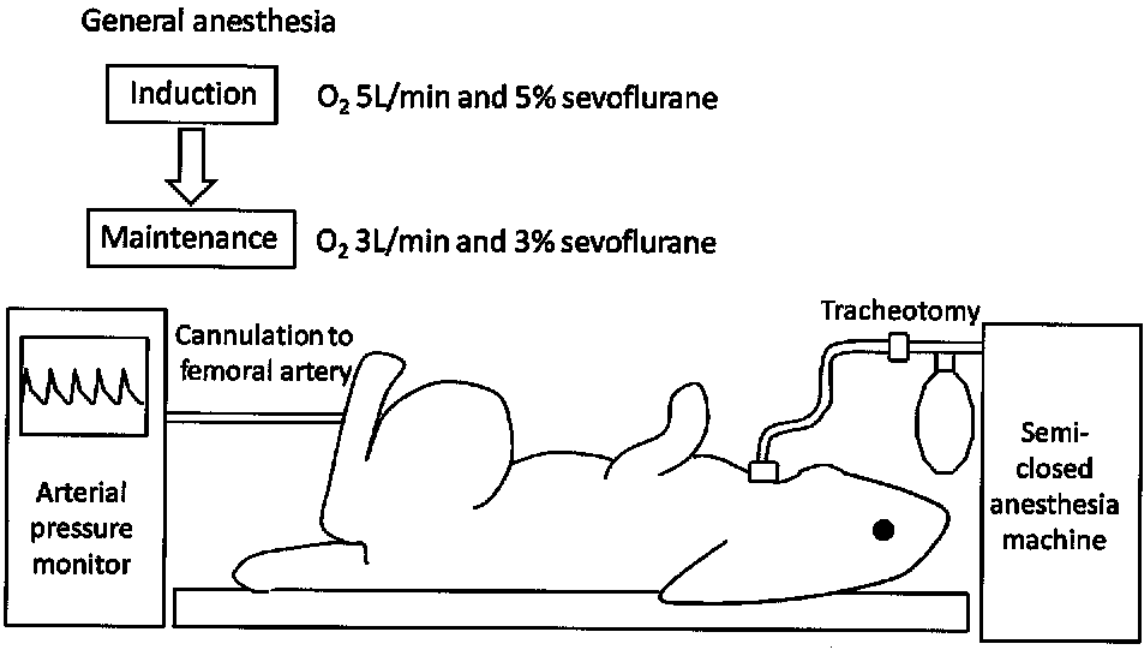

Figure 1. Method of general anesthesia

General anesthesia was induced by oxygen 5L/minute and 5% sevoflurane, and then a tracheotomy was performed, after which general anesthesia was maintained at oxygen 3L/minute and 3% sevoflurane. A cannula was inserted into the femoral artery, and arterial pressure was continuously recorded throughout the experiment using a polygraph and a pressure transducer.

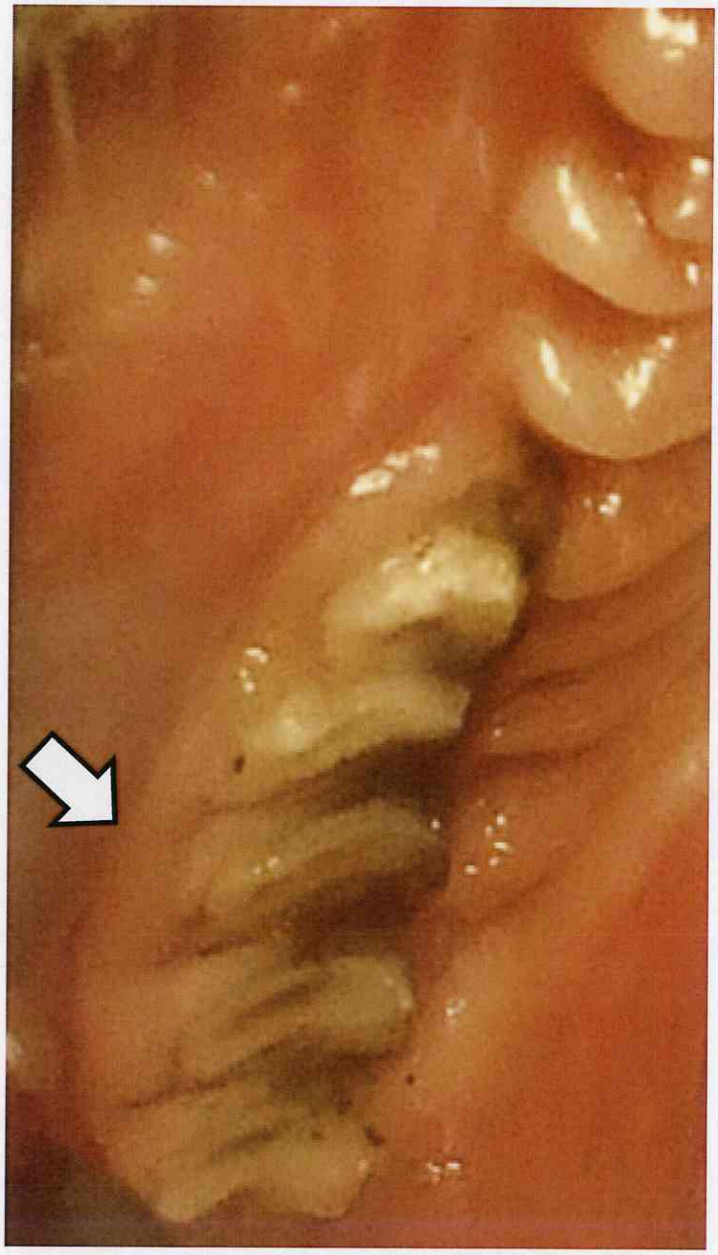

Figure 2. Location of infiltration anesthesia

0.5 mL of 2% of lidocaine containing 1/80000 adrenaline and 0.5 mL of 2% of adrenalin additive-free lidocaine was infused into the right maxillae, for 20 seconds, respectively. Injection site was the buccal side of the third molar on both sides (White arrow). Subperiosteal infiltration anesthesia was performed by touching the needle tip to the jaw bone surface under the periosteum.

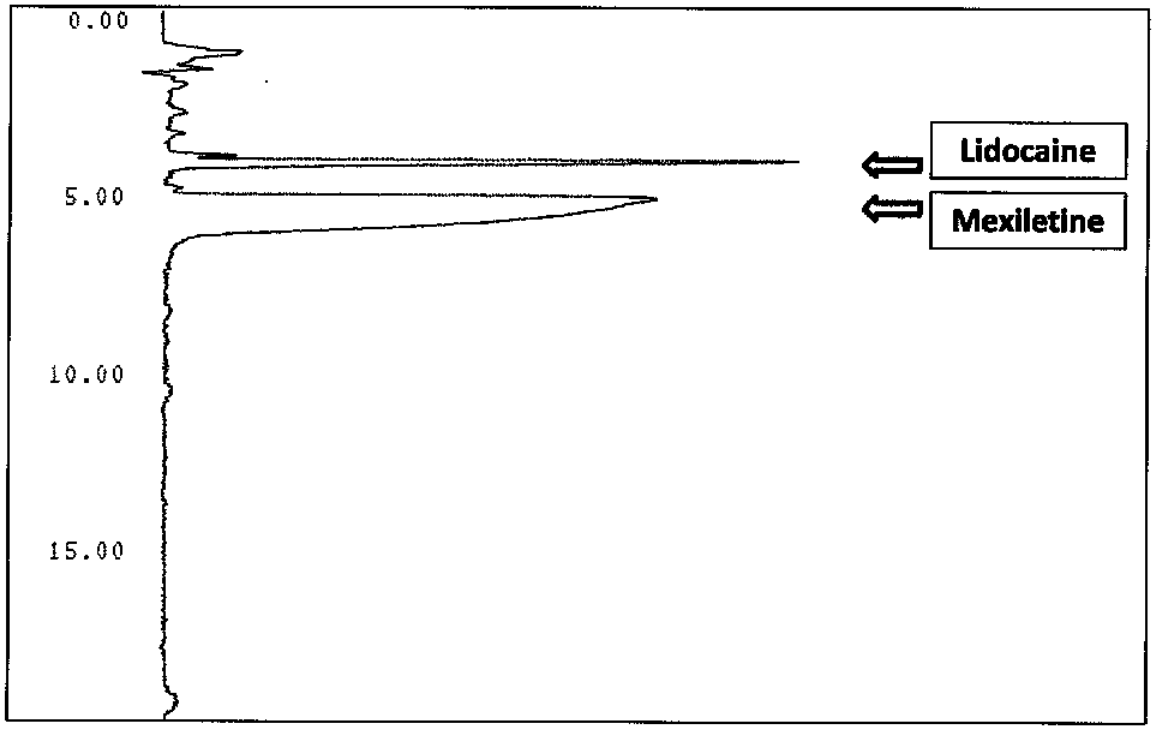

Figure 3. One case of chromatogram in lidocaine concentration from oral mucosa in rabbit

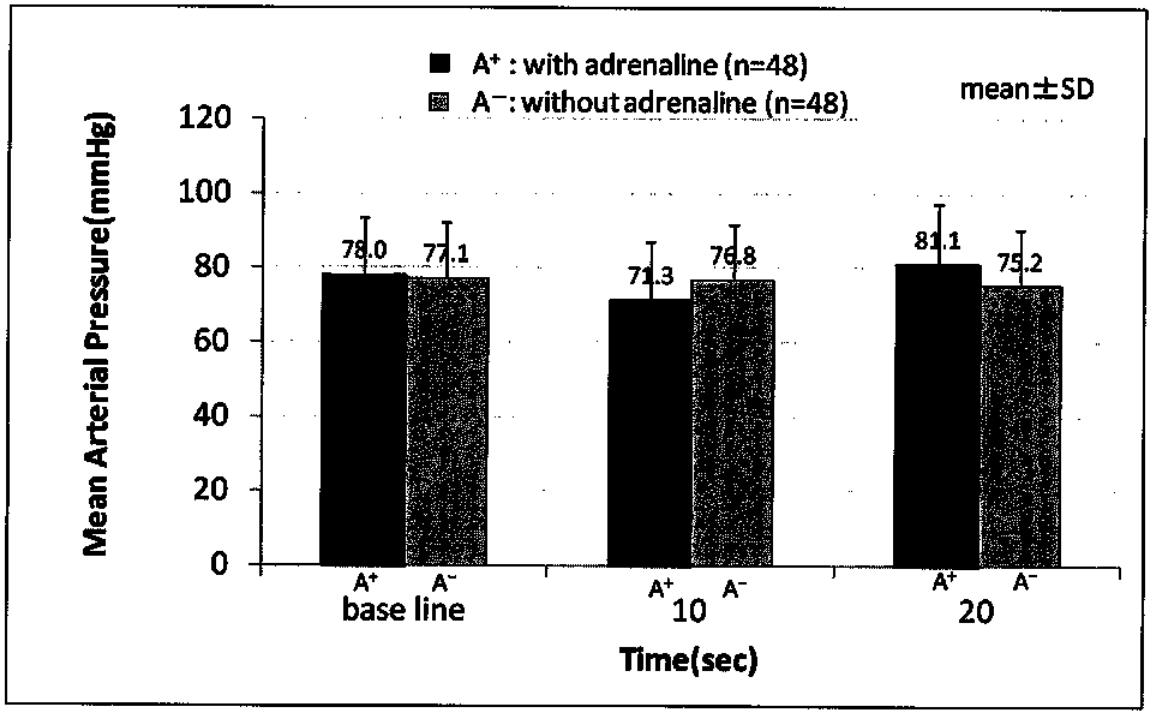

Figure 4. Change of mean arterial pressure before and after infiltration anesthesia

No significant difference was observed.

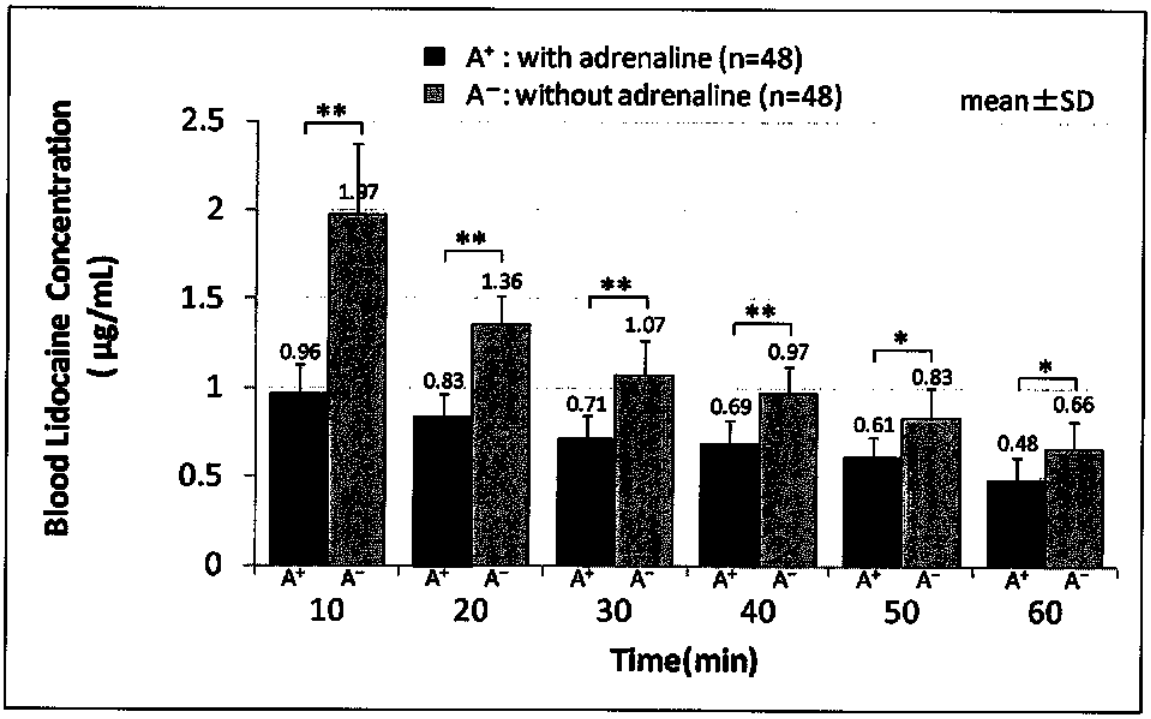

Figure 5. Change of blood lidocaine concentration after infiltration anesthesia

At all time-points, blood lidocaine concentration in A^+ was significantly lower than that in A^-.

** $P<0.01$ A^+ vs A^-

* $P<0.05$ A^+ vs A^-

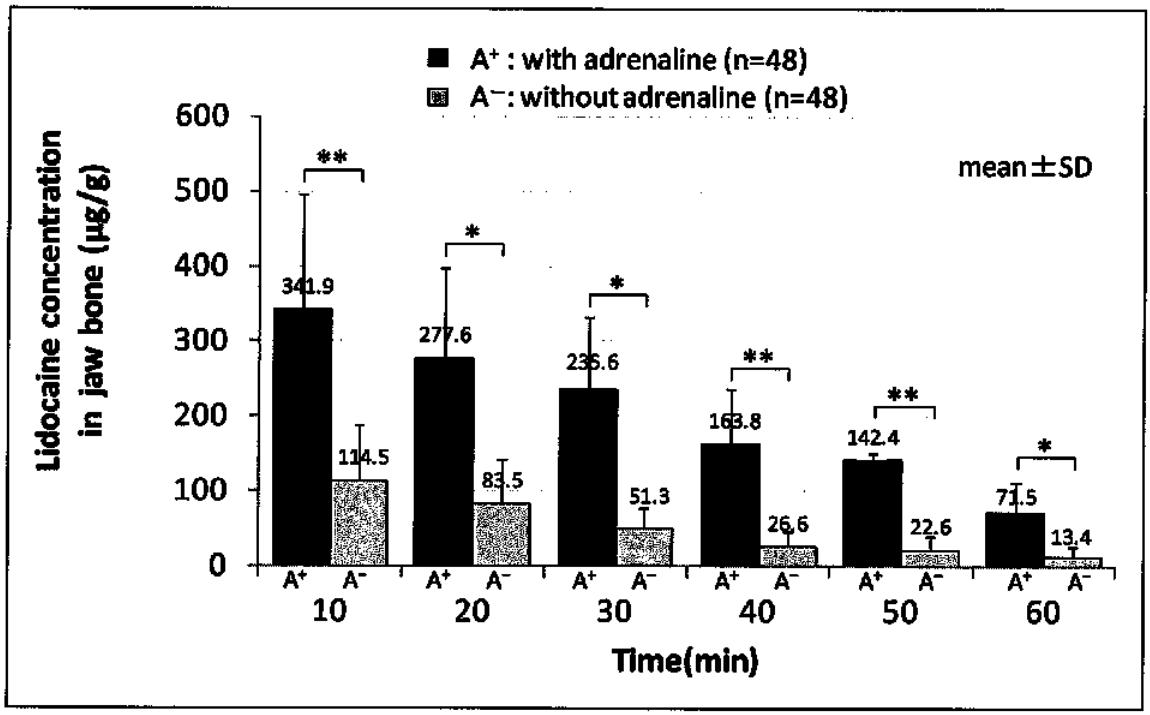

Figure 6. Change of lidocaine concentration in jaw bone after infiltration anesthesia

At all time-points, lidocaine concentration in jaw bone in A^+ was significantly higher than that in A^-.

** $P<0.01$ A^+ vs A^-

* $P<0.05$ A^+ vs A^-

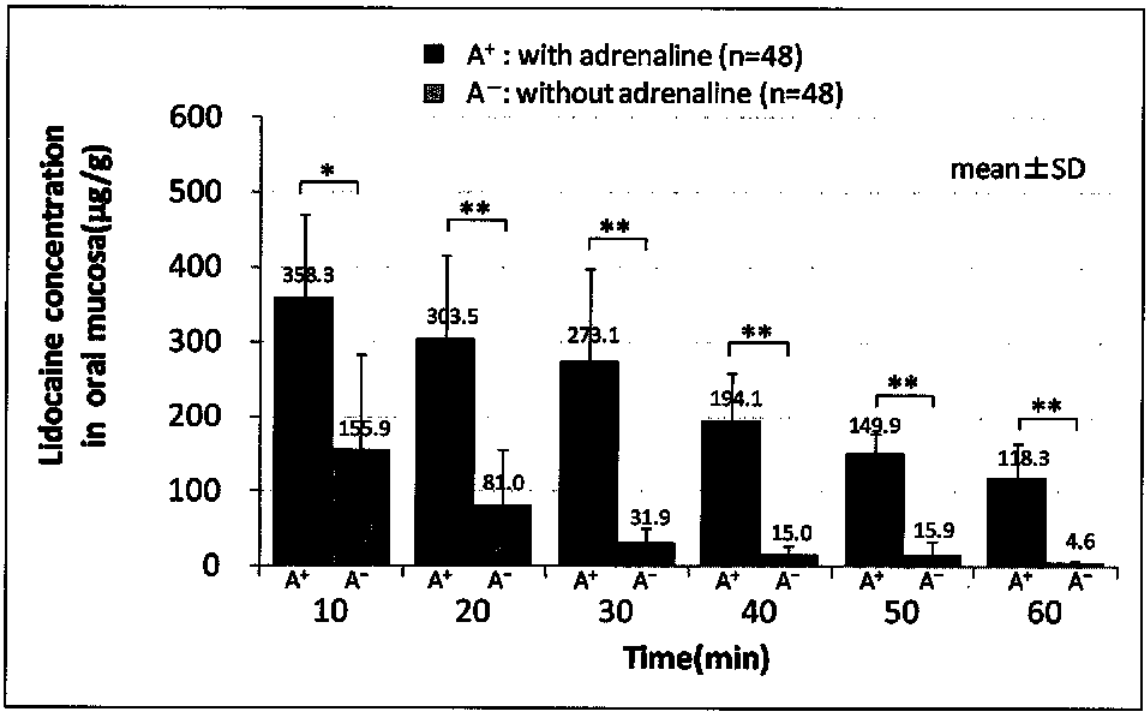

Figure 7. Change of lidocaine concentration in oral mucosa after infiltration anesthesia

At all time-points, lidocaine concentration in oral mucosa in A^+ was significantly higher than that in A^-.

** $P<0.01$ A^+ vs A^-

* $P<0.05$ A^+ vs A^-

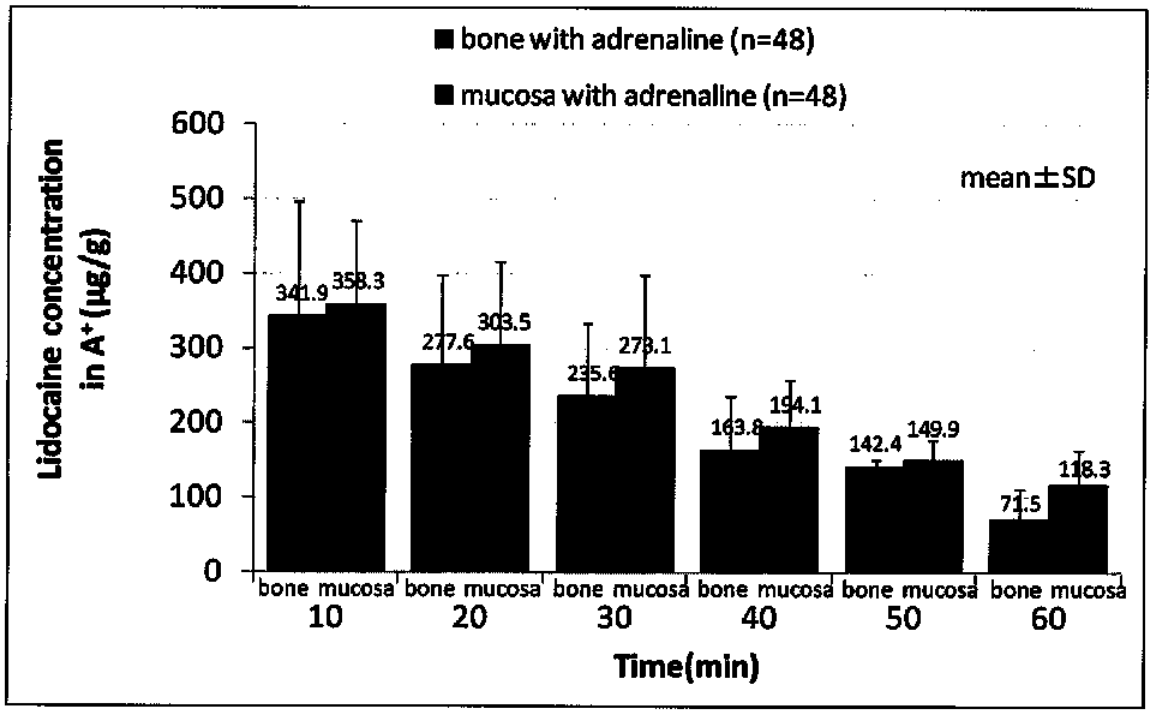

Figure 8. Change of lidocaine concentration in jaw bone and oral mucosa in A^+ after infiltration anesthesia

Lidocaine concentration in jaw bone was lower than that in oral mucosa at all time-points, and no significant difference was observed between the concentration values of both groups.

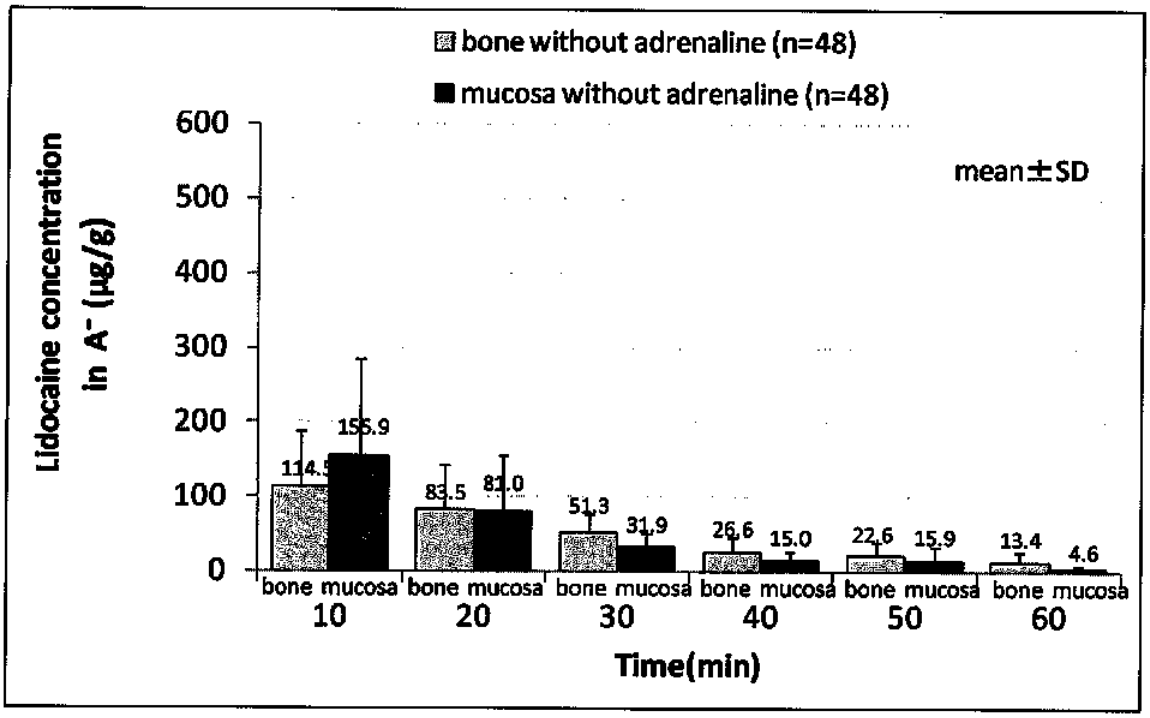

Figure 9. Change of lidocaine concentration in jaw bone and oral mucosa in A^- after infiltration anesthesia

Lidocaine concentration in jaw bone was lower than that in oral mucosa only at the 10-minute time point. Values thereafter were reversed, and no significant difference was observed between the two concentration values of both groups.